Research Article

Journal of Applied Toxicology

Received: 27 May 2008, Revised: 29 September 2008, Accepted: 1 October 2008, Published online 6 November 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/jat.1399

Vitamin A deficiency injures liver parenchyma and alters the expression of hepatic extracellular matrix

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ABSTRACT: Vitamin A is an essential lipid-soluble nutrient that is crucial for morphogenesis and adult tissue maintenance. The retinoid homeostasis in the liver depends on a regular supply of vitamin A from an adequate dietary intake to preserve the normal organ structure and functions. This study focuses on the effect of vitamin A deficiency on the morphology and extracellular proteins expression of the liver in adult Wistar rats. Animals were fed with a normal (control group) or deficient vitamin A diet for 3 months. At the end of the experimental period, histological examination of the livers under light and electron microscopy revealed that vitamin A deficiency produced a loss of hepatocyte cord disposition with an irregular parenchymal organization. Abundant fat droplets were present in the cytoplasm of the hepatocytes. Elongated myofibroblastic-like cells with an irregular cytoplasmic process and without lipid droplets could be seen at the perisinusoidal space, where an elevated intensity of alpha smooth muscle actin (α-SMA) was observed. These results suggest that an activation of hepatic stellate cells (HSCs) occurred. Moreover, immunochemical methods revealed that vitamin A deficiency led to an increased expression of hepatic fibronectin, laminin and collagen type IV. We propose that vitamin A deprivation caused liver injury and that HSCs underwent a process of activation in which they produced -SMA and synthesized extracellular components. These changes may be a factor predisposing to liver fibrosis. In consequence, vitamin A deprivation could affect human and animal health.
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Keywords: vitamin A; deficiency; extracellular matrix; liver; fibrosis

Introduction

Vitamin A is an essential lipid-soluble nutrient. This compound and its derivatives, retinol, retinal, retinoic and retinyl ester, have a profound influence during embryogenesis (Clagett-Dame and De Luca, 2002) and in adult tissues (Marín *et al*., 2005). Vitamin A and its derivatives participate in numerous cellular activities involved in reproduction, embryonic development, vision, growth, lipid metabolism, cellular differentiation, proliferation and tissue maintenance and can modulate the expression of extracellular matrix (ECM) proteins (Blomhoff and Blomhoff, 2006).

The liver, the major storage site for vitamin A, is responsible for its metabolism by regulating retinoids homeostasis (Blomhoff, 1994). The hepatic tissue is not composed solely of cells. A substantial part of its volume is an intercellular space filled with an intricate network of macromolecules secreted locally that constitute the ECM (Yamada, 1991; Rojind and Greenwel, 1994). Hepatic ECM includes several families of structural and supporting molecules: collagens, non-collagen glycoproteins, matrix-bound growth factors, proteoglycans and matrix cellular proteins (Schuppan *et al*., 2001). The ECM is a dynamic regulator of cell function, playing an active role in regulating the behavior of the cells. The ECM can also provide specific signals through interaction with the cell surface (Senoo and Hata, 1994a).

In normal livers, three cell types have been proved capable of the synthesis and secretion of ECM components: hepatocytes, endothelial cells and hepatic stellate cells (HSCs) (Clement *et al*., 1986). It is now known that these nonparenchymal quiescent cells are the primary source of ECM (Friedman, 2000).

The main function of HSC is to store vitamin A and its derivatives, retinoids, and probably to maintain the normal basement membrane-type matrix (Senoo and Hata, 1994b). Numerous *in vivo* and *in vitro* studies have shown that, when the liver is injured HSCs undergo a process of activation and differentiate into myofibroblasts (Iredale, 2001; Arnaud *et al*., 2003). Activation of HSC is characterized mainly by the loss of lipid droplets and vitamin A, production of alpha smooth muscle actin (α-SMA) and enhanced proliferation and synthesis of ECM components (Mann and Smart, 2002). Stellate cell activation leads to accumulation of scar (fibril-forming) matrix. This in turn contributes to the deterioration of the hepatic function, leading to fibrogenesis of the liver (Safadi and Friedman, 2002).

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The excessive depositions of ECM components in fibrotic livers result from an imbalance between the synthesis and degradation of matrix proteins (Martinez Hernandez and Amenta, 1993; Bedossa and Paradis, 2003). It is well known that HSCs are the main producer of collagen in normal and cirrhotic liver (Bataller and Brenner, 2005).

Hepatic fibrogenesis represents a wound-healing response of the liver to a variety of agents including toxins, disorders of the immune system, viral and parasitic infections, hemocromatosis and galactosemia (Leo and Lieber, 1999; Reeves and Friedman, 2002). However, the possibility that the excess or deficiency of essential nutrients such as vitamins can lead to hepatic alterations has scarcely been studied.

There is some information concerning the action of retinoids on the expression of ECM molecules and the reciprocal influence of these molecules on the response of surrounding cells (Kmiec, 2001; Axel *et al*., 2001). However, little is known about the effect of a vitamin A deficiency on the expression of liver ECM molecules (Scita and Wolf, 1994). Since the modification of ECM components is an unavoidable step in the progression of fibrogenesis, we here attempted to elucidate whether vitamin A deficiency is the trigger that starts this common and dynamic mechanism of fibrotic diseases.

In consequence, the aim of the present study was to analyze the toxic effect of vitamin A deprivation in a rat experimental model, studying the liver expression of fibronectin, laminin and collagen IV in association with α-SMA, a recognized marker of HSC activation, by immunohistochemistry, western blotting and electron microscopy. We also evaluated the histological appearance of livers from vitamin A-deficient rats

Materials and Methods

Diet and Experimental Design

Wistar rats were obtained from Romanelli SRL (Buenos Aires, Argentina), bred in our animal facilities and maintained at 21–23 °C in a controlled environment with a 12 h light–dark cycle. Female rats (45–50 g) were used in all experiments. They were weaned at 21 days of age and immediately assigned randomly to two groups (10 rats per group). The vitamin A-adequate (control) group was fed a standard AIN-93 diet (Reeves *et al*., 1993) while the vitamin A-deficient group was fed the standard AIN-93 diet without vitamin A. Rats were housed in individual cages and kept in a controlled environment. They were given free access to food and water throughout the 3 months of the experimental period. All experiments were conducted in accordance with NIH guidelines for the care and use of experimental animals (National Research Council, 1985). Both diets had the following composition (g/kg): 397.5 cornstarch, 100 sucrose, 132 dextrinized cornstarch, 200 vitamin-free casein, 70 soybean oil, 50 cellulose fiber, 35 AIN-93 mineral mix, 10 AIN-93 vitamin mix (devoid of vitamin A for the vitamin A deficient diet), 3 L-cystine, 2.5 choline bitartrate and 0.014 tert-butylhydroquinone. Body weight and food intake were registered daily.

Plasma and Liver Total Retinol Concentration Analyses

Ten rats per dietary groups were killed by cervical dislocation. Blood samples were collected in EDTA-coated tubes. The liver was separated and weighed and immediately thereafter its right upper lobe was removed for sampling. To minimize photoisomerization of vitamin A the plasma and liver samples were taken under reduced yellow light and frozen in the dark at −70 °C until determination of retinol concentrations. Analyses were carried out within 1–3 weeks of obtaining the samples. Plasma and liver retinoids were extracted using hexane and their total retinol concentration was determined by high performance liquid chromatography (HPLC) (Bieri *et al*., 1979; Mobarhan *et al*., 1986). As internal standard, retinyl acetate, was used. Chromatography was performed on a Nucleosil 125 C_{18} HPLC column with methanol:water (95:5, v/v) as the mobile phase. Retinol was detected by UV absorbance at 325 nm (model 440, Waters Associates) and peak areas were calculated by integration (Spectra Physics Analytical).

Histological Studies

A liver sample was immediately removed at necropsy and a portion of it was immersed in Bouin's fixative for histological and immunohistochemical studies. To evaluate histopathological changes in the liver of the vitamin A-deficient rats, the sections were stained by the Hematoxylin and Eosin (HE) staining method and by Mallory's triple stain technique, and examined under a Leitz photomicroscope. Photomicrographs of the stained sections were taken with a Leica camera.

Transmission Electron Microscopy (TEM)

Thin sections of livers were fixed for 4 h at 4 °C in 4% glutaraldehyde and 0.1% sodium phosphate (pH 7.4). Afterwards, specimens were washed twice in phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer at 4 °C overnight. Samples were dehydrated in an ethanol series and embedded in Spurr resin. Sectioning was carried out with a Potter Blum MT1 ultramicrotome. Slices were stained with lead citrate and uranyl acetate. Preparations were examined with a Zeiss EM electron microscope.

SDS-PAGE and Immunoblotting Procedure

For the sodium dodecylsulphate–polyacrylamide gel electophoresis (SDS-PAGE) and immunoblotting procedure, liver extracts were homogenized at 4 °C in extraction buffer (50 mmol/L Tris–HCl, pH 7.4, 0.1 mol/L NaCl and 1% Nonidet P-40), containing the following protease inhibitors: 2 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co., St Louis, MO, USA), 0.5 μg/mL leupeptin (Sigma), 5 μg/mL pepstatin (Sigma) and 5 μg/mL aprotinin (Sigma). Then, the extracts were centrifuged at 10 000***g*** for 5 min. Lipids were extracted from the supernatant as described by Heifetz and Snyder (1981) by adjusting the sample to 10 : 10 : 3 (v/v/v) chloroform–methanol–water. Precipitated proteins were solubilized in sample buffer for SDS–PAGE.

Denaturing PAGE was carried out following Laemmli's procedure (1970). SDS-denatured proteins were electrophoresed in 5 and 7.5% polyacrylamide gels. Samples were boiled in 2% SDS, 2% 2-mercaptoethanol, 20 mmol/L Tris–HCl, pH 7, for 3 min. The molecular weights of the denatured samples were estimated from a calibration curve obtained with the standard proteins in a pre-stained SDS–PAGE standard solution (Sigma). The gels were stained with Coomassie Blue R-250. Protein determination was performed by the method of Lowry *et al*. (1951).

After SDS–PAGE, the immunoblotting analyses were performed according to the method of Towbin (1979). Electrophoresed proteins were transferred from the gel to a nitrocellulose membrane rinsed with distilled water and blocked with 3% bovine serum

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albumin in phosphate buffer saline (BSA–PBS) for 1 h at room temperature. Dilutions, 1 : 100, of polyclonal antibody to human plasma fibronectin (Sigma, F3648) or of polyclonal antibody to laminin-1 (Sigma, L9393) or of monoclonal anticollagen type IV clone COL-94 (Sigma, C1926) were added and incubated overnight al 4 °C. Then the membranes were rinsed three times with PBS for 10 min with gentle shaking. The antigen–antibody reaction was detected using biotin-conjugated secondary antibodies and ExtrAvidin Peroxidase conjugates (Sigma); each of them were diluted 1 : 500, and the membranes were incubated for 2 h at room temperature. After each incubation, the membranes were rinsed three times with PBS for 10 min each time. Peroxidase activity was detected by incubating blots with 3,3'-diaminobenzidine–H_2O_2.

Western blots were scanned with the Gel Doc 1000 image scanner (Bio-Rad, CA, USA) and the band intensities were estimated and analyzed with an Image J 1.38 (NIH Image) software program.

Immunohistochemistry

Immunolocalization of the extracellular matrix. The localization of the ECM in liver sections of control and vitamin A-deficient rats was determined by the immunoperoxidase method described previously (Sánchez *et al*., 2001). Specimens fixed in Bouin's solution were dehydrated through a graded alcohol series and embedded in paraplast. The sections were cut into 7 μm-thick slices, deparaffinized and rinsed with PBS (pH 7.4). Then, they were incubated with 0.3% H_2O_2 in methanol for 30 min to inactivate the endogenous peroxidase and treated with 0.1% Trypsin (Merck KgaA, Darmstadt, Germany) for 10 min at room temperature to unmask antigenic sites of extracellular laminin in the basal lamina. Slices were incubated with 3% BSA–PBS for 1 h at room temperature with a 1 : 100 dilution of polyclonal antibody to human plasma fibronectin (Sigma, F3648) or with a 1 : 100 dilution of polyclonal antibody to laminin-1 (Sigma, L9393) or with a 1 : 100 dilution of monoclonal anticollagen type IV clone COL-94 (Sigma, C1926). After rinsing with PBS the sections were treated with a 1 : 500 dilution of biotinylated secondary antibodies for 2 h at room temperature and with ExtrAvidin-Peroxidase conjugate (Sigma) for 2 h at room temperature. Peroxidase activity was detected by incubation with 3,3'-diaminobenzidine (Sigma) in the same conditions for 10 min. The reaction was stopped by rinsing with distilled water and slices were mounted in Mowiol (Hoechst Verkauf Lackrohstoffe Frankfurt, Germany) and observed with a Nikon Fluophot microscope. Control sections for immunostaining, which consisted of the omission of the primary antibody or the substitution of the primary antibody by the corresponding nonimmune sera, were invariably negative.

Detection of alpha smooth muscle actin. Alpha smooth muscle actin was detected in sections of control and vitamin A-deficient rat livers. Briefly, liver tissue was deparaffinized with xylene and ethanol. After inactivation of the endogenous peroxidase, it was treated with 3% BSA-PBS, and incubated with the primary antibody a 1 : 100 dilution of monoclonal anti α-SMA clone1A4 (Sigma, A2547). After treating with 1 : 500 dilution of biotin-conjugated anti-mouse immunoglobulins (Sigma) and ExtrAvidin-Peroxidase conjugated, the reactive sites were detected using 3,3'-diaminobenzidine–H_2O_2, resulting in brown staining after incubation. In control slides, the primary antibody was substituted by 3% BSA-PBS.

Statistical Analysis

The numerical data, expressed as mean ± SEM, were statistically evaluated using Student's *t*-test at a 5% level of significance.

Results

None of the rats died before the termination of the study and no observable deleterious side effects resulted from vitamin A deficiency. There was no difference in the average daily food intake between controls and vitamin A-deficient rats and no difference in body weight was observed between the two groups throughout the experiment (data not shown).

Plasma and Liver Total Retinol Concentration Analyses

After the 3 months of the experimental period, the plasma and liver retinol levels of vitamin A-deficient rats (0.60 ± 0.11 μmol/L, $P < 0.005$; 0.05 ± 0.002 μmol/L, $P < 0.001$, respectively) were significantly lower than those of control animals (2.03 ± 0.21 and 1.76 ± 0.10 μmol/L, respectively). Values are the mean ± SEM of 10 rats per dietary group. The retinol concentrations in the plasma and liver of the vitamin A-deficient rats were 71 and 97% lower than in the controls, respectively.

Histology and Ultrastructural Analysis

In contrast to control livers [Fig. 1(A)], vitamin A-deficient livers stained with HE had irregularly distributed hepatocytes without a characteristic cord disposition. Their nuclei showed varying sizes and shapes with an irregular chromatin distribution. The cytoplasm of the hepatocytes contained a large number of fat droplets, which reached a significant size, leading to a reduction in the cytoplasm and a displacement of the nucleus towards the periphery [Fig. 1(B)]. Vitamin A-deficient rats had perisinusoidal spaces filled with extracellular materials, easily identified by Mallory's triple stain [Fig. 1(D)].

In control animals, transmission electron microscopy revealed a normal ultrastructure of the hepatocytes with a round clear nucleus [Fig. 2(A)]. They were characterized by an elevated number of large mitochondria. The abundant rough endoplasmic reticulum surrounded the mitochondria and was in close contact with them [Fig. 2(B)]. Transverse sections of the smooth endoplasmic reticulum associated with glycogen granules and characteristic lysosomes were also observed.

The HSC appeared in contact with hepatocytes. It showed a characteristic non-proliferative phenotype, with tight nuclear chromatin peripherally disposed and contained several fat droplets, which occupied the larger part of the cytoplasm [Fig. 2(A)].

In contrast, vitamin A-deficient livers showed hepatocytes with altered ultrastructural characteristics, consisting of a dilated rough endoplasmic reticulum. Ribosomes appeared more scattered and without a regular structure, more electrondense mitochondria and abundant lipid droplets dispersed in a condensed cytoplasm [Fig. 3(A)].

Vitamin A-deficient livers showed cells with ultrastructural characteristics of activated HSC. These cells exhibited a significant loss of fat droplets. They appeared elongated with irregular cytoplasmic processes of dendritic configuration such as myofibroblasts. Collagen fiber units disattached by exocytosis from the activated HSC could be observed [Fig. 3(B)]. The presence

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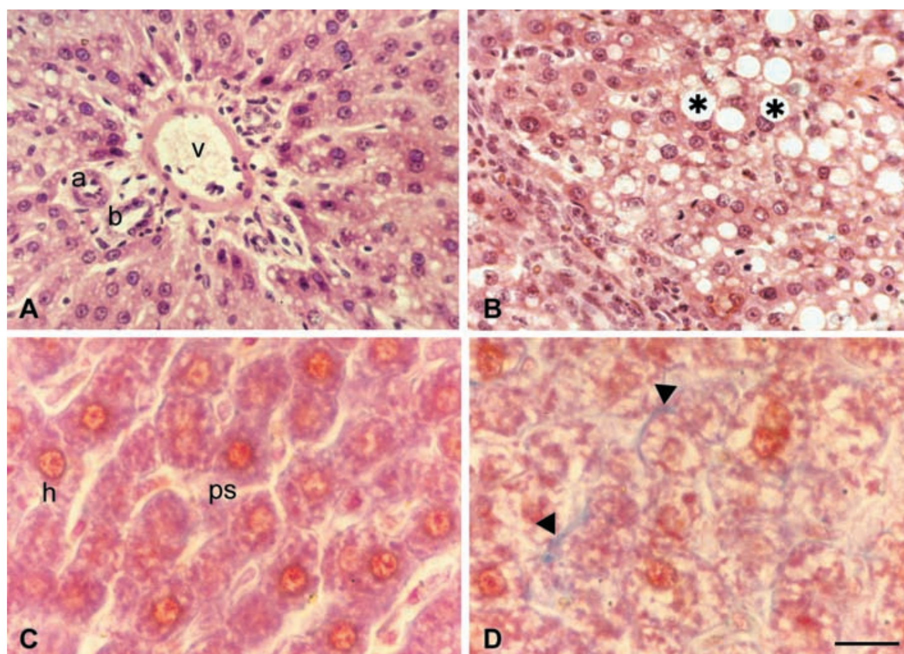

Figure 1. Hematoxylin–Eosin and Mallory's triple staining of liver sections. Hematoxylin-Eosin stained sections A–B. (A) Normal histoarchitecture and cordonal parenchymal organization can be observed. Hepatocytes presented round nuclei with one or two prominent nucleoli. a, arteriole; b, bile duct; v, central vein. (B) Vitamin A-deficient liver shows the presence of abundant fat droplets in the hepatocytes (*). Nuclei with varied sizes and shapes can be observed. Note the irregular arrangement of the hepatic cords. Bar: 50 μm. Photographs are representative of all sections studied. Mallory's triple stained sections C–D. (C) Control section. Its appearance is normal. (D) Vitamin A-deficient rat livers show enhanced extracellular materials stained with blue (arrowheads). h, hepatocytes; ps, perisinusoidal space. Bar: 25 μm. Photographs are representative of all sections studied.

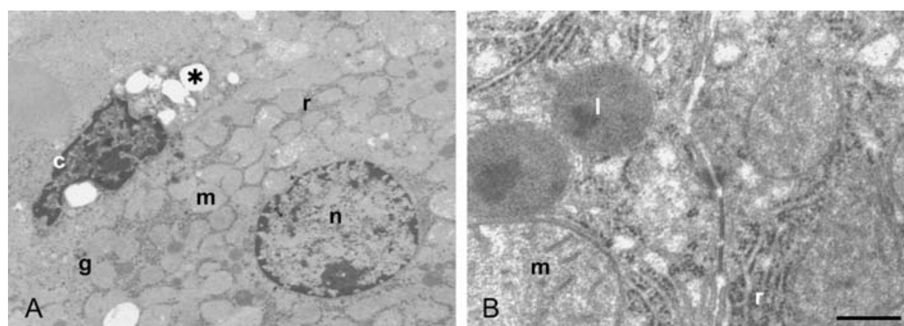

Figure 2. Electron micrograph of normal liver section. (A) The hepatocyte shows a round nucleus (n), numerous large mitochondria (m), an abundant rough endoplasmic reticulum (r) and glycogen particles (g). HSC is in close contact with the hepatocyte. Nuclear chromatin is disposed in the periphery (c). Fat droplets occupy most of the cytoplasm (*). Bar: 3 μm. (B) Higher magnification of the hepatic cytoplasm shows a characteristic distribution of the rough endoplasmic reticulum (r), lysosomes (l) and mitochondria. Bar: 0.9 μm.

of a great amount of extracellular materials such as collagen was observed in the vicinity of the myofibroblastic cells [Fig. 3(C)].

Expression of Fibronectin and Laminin-1 in Liver Extracts

In order to determine the expression of fibronectin, laminin and collagen IV in vitamin A-deficient and control rats, liver tissue extracts were subjected to western immunoblotting (Fig. 4). Blot analyses revealed an increased expression of fibronectin, laminin and collagen IV protein levels in the livers of vitamin A-deficient rats compared with control animals (fibronectin 280.45 ± 8.47%; laminin 202.58 ± 12.04% and collagen IV; 165.09 ± 41.05%; comparative to control 100%, $P < 0.001$).

Immunolocalization of Fibronectin, Laminin-1 and Collagen IV in the Liver Tissue

The distribution of fibronectin was detected by immunoperoxidase labeling in liver sections. The major sites of deposition of fibronectin were found to be the perisinusoidal space, where the intensity of fibronectin immunoreactivity in the vitamin A-deficient rats was higher than in the controls (Fig. 5).

The distribution of laminin-1 was detected using its specific antibody. In control liver laminin staining only appeared in the portal area; however, in vitamin A deficiency laminin-1 was also expressed at the perisinusoidal space. The relative intensity of the labeling was in general higher in the vitamin A-deficient rats

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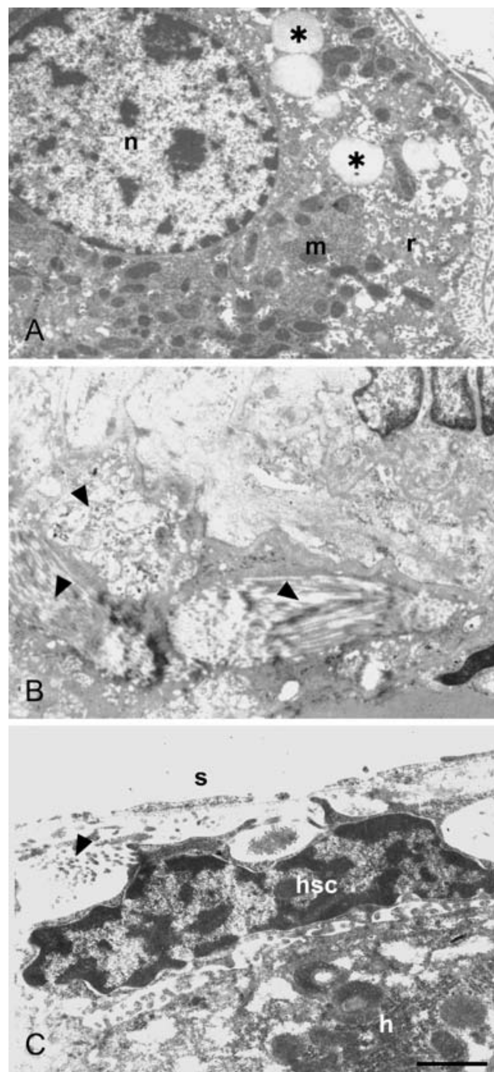

Figure 3. Electron micrograph of vitamin A-deficient liver section. (A) The hepatocyte shows an altered ultrastructure. A considerable part of the cytoplasm is filled with fat droplets (*). Note numerous electron dense mitochondria (m) and the dilated rough endoplasmic reticulum (r). Bar: 3 μm. (B) The presence of extracellular materials such as collagen can be observed (arrowheads). (C) An activated HSC cell with myofibroblastic characteristics can be observed. The cell appears elongated with irregular cytoplasmic processes and loss of fat droplets. Note the collagen fiber units disattached from the activated HSC (arrowheads). Bar: 3 μm. h, hepatocytes; hsc, hepatic stellate cell; s, sinusoid.

than in the controls (Fig. 6). The immune staining of collagen IV showed an increased expression of this protein at the perisinusoidal space compared with the control (Fig. 7).

These studies evidenced the presence of an increased amount of fibronectin, laminin and type IV collagen at the perisinusoidal space of vitamin A-deficient livers and agreed with western blot analyses.

Alpha Smooth Muscle Actin Detection in Liver Sections

In normal control group, α-SMA positive cells were observed at the portal space and around the central vein but were not detected along the sinusoids [Fig. 8(A)]. In the vitamin A-deficient group,

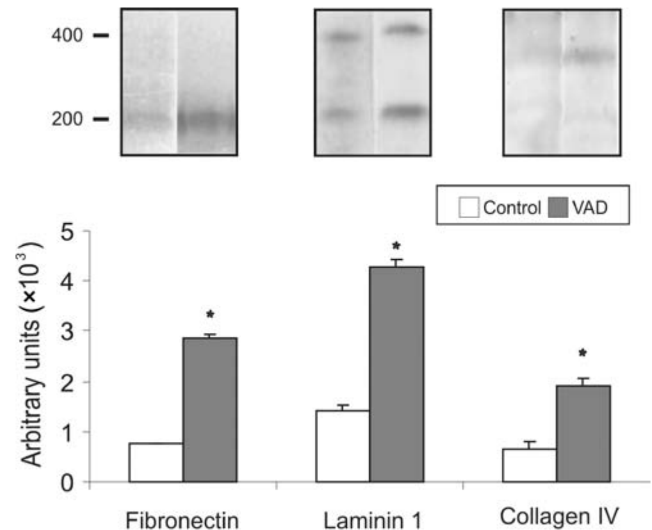

Figure 4. Quantification by western blotting of fibronectin, laminin and collagen IV in liver extracts from normal and vitamin A-deficient rats. Histograms represent in arbitrary units the densitometric scanning of the bands. Values are means ± SD, $n = 3$. $P < 0.001$. Inserts show representative western blots of fibronectin, laminin and collagen IV. The molecular weights at the detected protein bands are indicated.

α-SMA expression was greatly elevated and mainly detected along the sinusoids at the perisinusoidal space where the myofibroblastic cells were located [Fig. 8(B)].

Discussion

The main purpose of our study was to analyze the effects of vitamin A deficiency on hepatic extracellular matrix and microscopic liver morphology. The results indicated that vitamin A deficiency leads to: (1) liver ECM alteration with increased fibronectin, laminin and collagen IV expression; (2) hepatic architectural distortion and parenchymal disorganization with fat droplets in the hepatocyte cytoplasm; and (3) the presence of myofibroblastic α-SMA positive cells at the perisinusoidal space associated with ECM accumulation. These pathological effects of vitamin A deficiency did not seem to be due to malnutrition since there was no difference in the average food intake or average body weight between rats fed the vitamin A-deficient diet and those fed the vitamin A-adequate diet.

Vitamin A is an essential lipid soluble dietary precursor for the biosynthesis of at least two critical metabolites: retinal, required for the vision proteins (Blomhoff *et al*., 1991), and retinoic acid, a potent signaling molecule capable of altering the transcriptional activation or repression of numerous genes (Faraooqui *et al*., 2004). In view of this fact it is not surprising that both an excess and a deficiency of vitamin A can adversely affect human or animal health.

The liver, as several other organs, depends on a regular supply of vitamin A from several sources, and is the main store site for this nutrient (Goodman *et al*., 1966). The liver retinoids metabolism is closely integrated with peripheral tissues through the interorgan transfer and re-cycling of retinoids, affecting the whole-body economy of vitamin A (Ross and Zolfaghari, 2004). Thus, any condition that interferes with the ingestion, absorption, storage, release, transport or cellular uptake of vitamin A can

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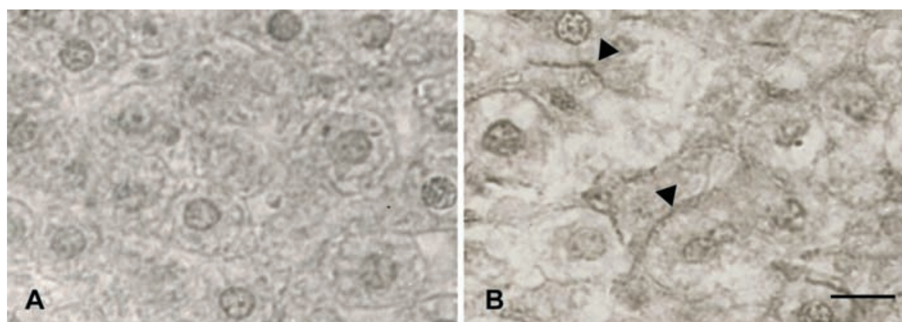

Figure 5. Immunolocalization of fibronectin in normal and vitamin A-deficient liver. (A) Control sections: no positive reactions are observed. (B) In vitamin A-deficient liver strong immunoreactivity (arrows) can be observed at the perisinusoidal space. Bar: 25 μm. Photographs are representative of all sections studied. This figure is available in colour online at www.interscience. wiley.com/journal/jat

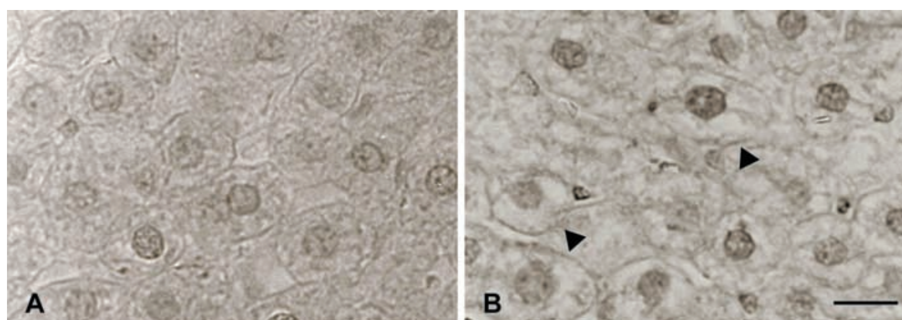

Figure 6. Immunolocalization of laminin in normal and vitamin A-deficient liver. (A) Control sections: no positive reactions are observed. (B) In vitamin A-deficient liver strong immunoreactivity (arrows) can be observed at the perisinusoidal space. Bar: 25 μm. Photographs are representative of all sections studied. This figure is available in colour online at www.interscience. wiley.com/journal/jat

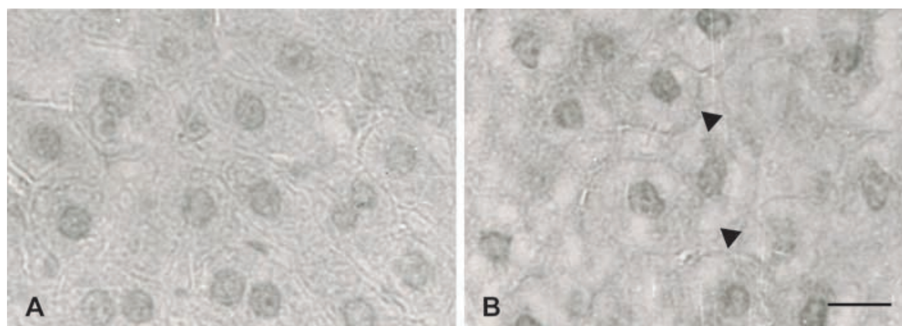

Figure 7. Immunolocalization of collagen IV in normal and vitamin A-deficient liver. (A) Control sections: no positive reactions are observed. (B) In vitamin A-deficient liver strong immunoreactivity (arrows) can be observed at the perisinusoidal space. Bar: 25 μm. Photographs are representative of all sections studied. This figure is available in colour online at www.interscience. wiley.com/journal/jat

induce deficiencies in target tissues (Biesalski *et al*., 1999; Oliveros *et al*., 2007)

Our results showed that experimentally induced vitamin A deficiency is associated with changes in the expression of fibronectin in rat livers. We observed a marked increase in fibronectin immunoreactivity at the perisinusoidal space. These findings agreed with previous works showing that vitamin A-deficient rats had increased levels of fibronectin in their serum (Zerlauth *et al*., 1984) and increased levels in liver fibronectin mRNA as observed by Omori and Chytil (1982) and Kim and Wolf (1987). These authors proposed that one of the functions of vitamin A is to act at the genomic level and that its deficiency alters genomic expression of fibronectin in the liver and in the hepatocytes. Our results support the idea that vitamin A could regulate the synthesis of fibronectin, increasing its expression in the liver.

In contrast, in the eyes of vitamin A-deficient rats this deprivation is associated with a decrease in endogenous fibronectin accompanied by delayed epithelial migration (Watanabe *et al*., 1991; Frangieh *et al*., 1989).

In the present study we also demonstrated that laminin-1 showed a different pattern of distribution in vitamin A-deficient rats compared with the control animals. In vitamin A-deficient

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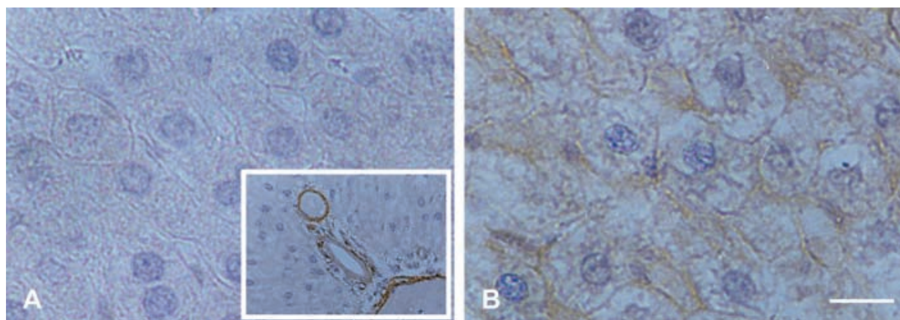

Figure 8. Distribution of α-SMA. (A) In control rats, perisinusoidal spaces are negative for α-SMA. α-SMA is found around the portal vessel (insert). (B) In vitamin A-deficient liver α-SMA is found along the perisisusoidal space. Bar: 25 μm. Photographs are representative of all sections studied.

rats laminin-1 is immunolocalized along the liver perisinusoidal space apart from its classic presence at the portal and central vein regions. A similar localization for laminin-1 has been described by Matsumoto *et al*. (1999) in liver fibrosis diseases. We suggest that the synthesis and deposit of laminin-1 in the perisinusoidal space could be a first step in fibrogenic processes associated with vitamin A deficiency.

In normal liver collagen IV with laminin-1 takes part in the formation of a low-density basement membrane-like material along the sinusoidal wall, which is critical for the easy diffusion between blood and liver cells and for maintaining the differentiated function of neighboring liver cells such as hepatocytes and sinusoidal cells (Bedossa and Paradis, 2003).

In vitamin A-deficient rats collagen IV exhibited an increased staining at the perisinusoidal space together with an increased laminin-1 expression. The accumulation of both ECM proteins modifies the Disse space. Interestingly, in advanced stages of fibrosis an electron-dense basal membrane was observed along the sinusoid wall (Neubauer *et al*., 2001).

In spite of the increased collagen expression observed in the liver of our experimental animals, reduced collagen was described in the adventitia of small caliber pulmonary arteries and arterioles and in the alveolar septa in the lung of vitamin A-deficient rats (Baybutt *et al*., 2000). Moreover, no changes in type III collagen were found in lung retinol-deficient rats (Veness Meehan, 1997). At the renal level Marín *et al*. (2005) showed that chronic vitamin A deficiency during the growth period altered the structure and collagen IV composition of rat renal basement membranes at both the mRNA and the protein levels.

In our experimental conditions vitamin A deficiency produced an enhanced expression of hepatic fibronectin, laminin and collagen IV. This altered ECM expression was accompanied by morphological alterations. Histological studies showed that vitamin A deficiency produced loss of hepatocytes cord disposition, with fat droplets accumulation in the cytoplasm of these cells. At the ultrastructural level, we observed transformed, α-SMA-positive HSC. These findings suggest the possibility that an activation of HSC could take place. HSC, the main cellular source of ECM during chronic liver injury, undergoes a transition into α-SMA expressing myofibroblast-like cells in response to various liver injuries such as cytokine, ethanol-mediated inflammation, oxygen free radical, lactic acid (Zhang *et al*., 2006), carbon tetrachloride widely used as a rodent model of fibrosis (Neubauer *et al*., 1999). Furthermore, HSC activation is associated with HSC proliferation, increased contractility and enhanced matrix production (Friedman, 1999). Therefore, HSC plays a crucial role in cellular and molecular events leading to hepatic fibrosis.

On the other hand, in association with α-SMA expression, we found extensive deposition of ECM in vitamin A-deficient liver. The intensity of fibronectin immunoreactivity was higher in the perisinusoidal space of vitamin A-deficient animals. Lui *et al*. (2000) suggested that the sinusoids and the perisinusoidal space are the original sites of fibrosis. Considering the fact that the deposition of fibronectin is an early stage in tissue healing and repair and seems to function as a template for deposition of other ECM proteins such as collagen type I and laminin-1 in injured areas (Gillis and Nagy, 1997), our findings would support the role of fibronectin as a pacemaker of fibrosis as suggested by Ritcher *et al*., (1998).

Although there are multiple etiologies and different forms, all types of liver fibrosis have in common the disorganization of the parenchyma (Friedman, 1993; Gressner, 1992; Bissell, 1998) and the excessive accumulation of ECM (Neubauer *et al*., 2001). Similarly, in our experimental conditions vitamin A deficiency produced both effects in the liver.

Interestingly, recent evidence suggests that liver fibrosis and even cirrhosis could be reversed, but the processes involved are unclear at this time. In an experimental model of liver fibrogenesis in mice, Wang *et al*. (2007) observed that retinoic acid, a potent metabolite of vitamin A, suppressed the type I collagen production in liver, reducing the hepatic injury caused by oxidative stress. Retinoic acid is a potent antioxidant, and its presence is necessary to alleviate the liver injury mediated by reactive oxygen species (Tamiguchi *et al*., 2004). Therefore, some authors (Elsharkawy *et al*., 2005; Friedman and Bansal, 2006; Purohit and Brenner, 2006) have suggested that the reduction in the number of HSCs through apoptosis is a key step in the antifibrotic process, even if the factors involved in HSCs apoptosis during liver fibrosis are not fully understood. In this regard, Radaeva *et al*. (2007) have demonstrated that during liver fibrosis activated HSCs become susceptible to natural killer (NK) cell killing, which may be an important mechanism contributing to HSC apoptosis. Also, these authors suggested that retinoic acid plays an important role in the sensitization of HSCs to NK cell killing. However, the underlying mechanisms remain unknown.

Our results suggest that vitamin A deficiency could be a signal that modulates the activation and transformation of HSC into myofibroblast, increasing ECM synthesis. These events could be the link between vitamin A deficiency and fibrogenesis, an unavoidable step in the progression of all chronic hepatopathies.

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Acknowledgements

This research was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT), Argentina; grants to Sara Sánchez. We wish to thank Stella Honoré, PhD, for her photographic assistance, and Ms Virginia Méndez for her proof-reading.

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